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Structure and biological activity of chemically modified nisin A species

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Nisin, a 34-residue peptide bacteriocin, contains the less common amino acids lanthionine, β-methyllanthionine, dehydroalanine (Dha), and dehydrobutyrine (Dhb). Several chemically modified nisin A species were purified by reverse-phase HPLC and characterized by two-dimensional NMR and electrospray mass spectrometry. Five constituents, [2-hydroxy-Ala5]nisin, [Ile4-amide,pyruvyl-Leu6]des-Dha5-nisin, [Met(O)21]nisin, [Ser33]nisin, and nisin-(1–32)-peptide amide, were found in a commercial nisin sample. A further species, [2-hydroxy-Ala5]nisin-(1–32)-peptide amide, was obtained by freeze drying an acidic nisin solution. These compounds are formed by chemical modification of nisin: the addition of a water molecule to the dehydroalanine residues, which can lead to the cleavage of the polypeptide chain, or the oxidation of methionine residues.

The 2-hydroxyalanine-containing products have a limited stability; they are spontaneously converted into the corresponding des-dehydroalanine derivatives. The growth-inhibiting activity of the modified nisins towards different bacteria was determined. The 2-hydroxyalanine-containing species and the des-dehydroalanine derivative show a strong reduction in biological activity as compared to native nisin. [Met(O)21]nisin and [Ser33]nisin show moderate or no reduction in biological activity.

Keywords: chemically modified nisin; lantibiotic; mass spectrometry; nisin; NMR.

Nisin is a small peptide bacteriocin produced by several *Lactococcus lactis* strains (Sahl et al., 1995). Nisin belongs to the group of lantibiotics and contains a number of uncommon amino acids, which are formed in a post-translational modification process. In this process, the unsaturated amino acids dehydroalanine (Dha) and dehydrobutyrine (Dhb) are formed from serine and threonine residues, respectively. Subsequently, specific addition reactions occur between cysteine residues and some of the unsaturated amino acids, which results in the formation of lanthionine and β-methyllanthionine residues. The thio-ether bridges of the lanthionines form intramolecular cross-links resulting in five cyclic structure elements in the molecule (Fig. 1).

Since nisin is active against a broad range of gram-positive microorganisms and is produced by food-grade bacteria it has been applied as a natural preservative in the dairy and the canning industries (Delves-Broughton, 1990). The exact molecular mechanism of the growth-inhibiting effect of nisin is still not fully understood, although several studies have found indications that nisin interacts with the bacterial cell membrane forming pores ultimately leading to cell lysis (Hurst, 1981; Gao et al., 1991; Driessen et al., 1995; Sahl et al., 1995).

The three-dimensional structure of nisin was elucidated by two-dimensional NMR techniques in aqueous solution (van de Ven et al., 1991; Lian et al., 1992) and in membrane-mimicking systems (van den Hooven et al., 1996a). Although the structure of nisin is different in the two media, the global characteristics are similar. While considerable flexibility is observed in the part connecting the third and fourth ring, the structure of the smaller rings is relatively well defined. The molecule has an amphiphilic character, which has been proposed to be important for its biological function (van de Ven et al., 1991; van den Hooven et al., 1996a,b).

Due to the reactivity of the Cα-Cβ double bond, the unsaturated amino acids in nisin are potential sites for chemical modification which limits the chemical stability of nisin. It appears that in particular dehydroalanine residues are susceptible to acid-catalysed addition of a water molecule to the double bond (Gross, 1977). This first leads to the formation of a 2-hydroxyalanine residue at which the polypeptide chain subsequently is cleaved into an N-terminal peptide amide and a C-terminal pyruvyl peptide (Fig. 2). Two chemically modified nisin A species, nisin-(1–32)-peptide amide and [Ile4-amide,pyruvyl-Leu6]des-Dha5-nisin-(1–32)-peptide amide, which were formed according to this mechanism, were isolated and characterized by two-dimensional NMR techniques (Chan et al., 1989; Lian et al., 1992). It appeared that the removal of the two C-terminal residues does not affect the biological activity. The modification of Dha5 followed by the opening of the first ring next to the N-terminus strongly reduces the biological activity as compared to intact nisin (Chan et al., 1989). This indicates that Dha5 or an intact ring A is essential for the functional properties of nisin. It has been reported that [Ala5]nisin and [Ala5, Ala33]nisin have normal biological activities (Dodd and Gasson, 1994; Kuipers et al., 1996). [Ala5]subtilin, a bacteriocin structurally very similar to nisin, was reported to have an unimpaired growth-inhibiting activity but a strongly reduced spore-killing effect (Liu and Hansen, 1992). [Dhb5]nisin Z showed considerable reduction in activity, although a dehydrated residue was preserved in this position (Kuipers et al., 1992). In the present paper, we report the isolation and characterization of five chemically modified

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Abbreviations. Dha, dehydroalanine; Dhb, dehydrobutyrine; ESI, electrospray ionization; MIC, minimal inhibitory concentration; RP, reverse phase.

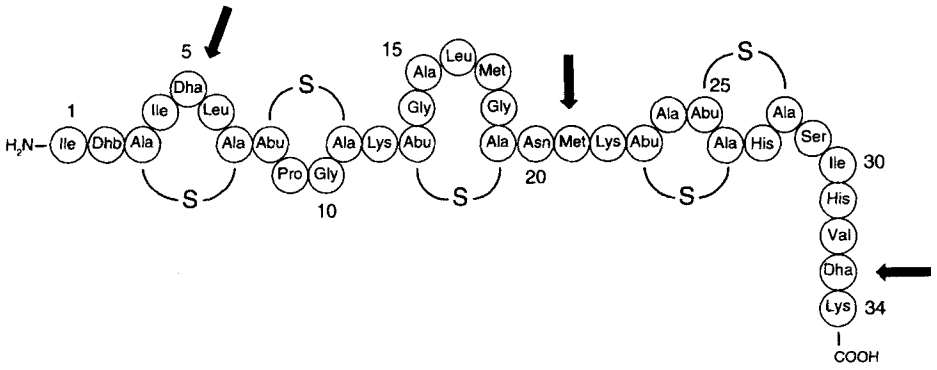

Fig. 1. Schematic representation of the primary structure of nisin A. Ala-S-Ala represents lanthionine, Abu-S-Ala β-methyllanthionine, Dha dehydroalanine, and Dhb dehydrobutyrine. Arrows indicate the sites of modification.

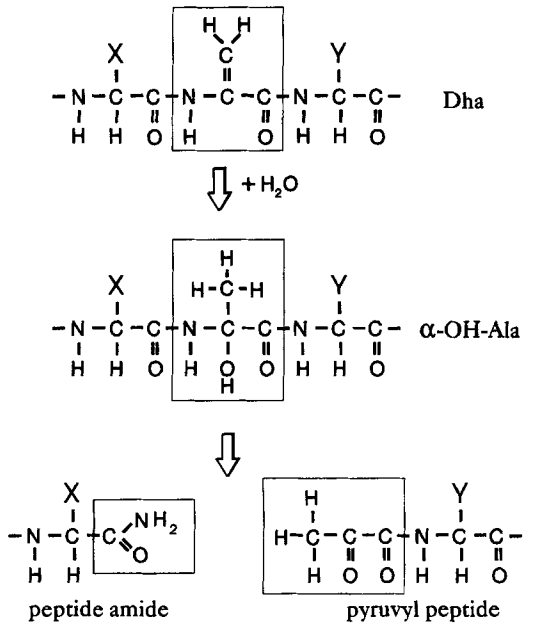

Fig. 2. Schematic representation of the acid-catalysed addition of a water molecule to a Dha residue (boxed), leading to the cleavage of the polypeptide chain.

nisin species originating from a commercial nisin sample and one compound resulting from freeze drying of an acidic nisin solution. A preliminary report of a part of this study has appeared elsewhere (Rollema et al., 1991). The chemical structures of the nisin species were determined by two-dimensional NMR and electrospray ionization mass spectrometry (ESI-MS), and their growth-inhibiting activity towards different bacterial strains was assayed.

MATERIALS AND METHODS

Materials. The modified nisins were isolated by RP-HPLC (reverse-phase HPLC) from a commercial nisin sample (Aplin & Barrett). [2-hydroxy-Ala5]nisin-(1–32)-peptide amide was prepared by freeze drying of a 1 mg/ml solution of nisin in 0.1 M HCl followed by RP-HPLC purification. The solvent was removed from the isolated nisin fractions by rotary evaporation. The nisin derivatives were dissolved in 0.05 % acetic acid and freeze dried.

RP-HPLC. The HPLC equipment consisted of a Gilson 231 automatic sample injector, two Waters 510 pumps, and a Waters Lambda-Max 481 variable-wavelength detector. Bio-Rad Hi-Pore RP318 columns were used: 250 mm×21.5 mm for preparative runs (flow 10 ml/min) and 250 mm×4.5 mm for analytical runs (flow 1 ml/min). As eluents, 10 % aqueous CH_3CN/0.1 % CF_3CO_2H (buffer A) and 90 % aqueous CH_3CN/0.07 % CF_3CO_2H (buffer B) were used. A typical gradient used in analytical separations was from 23 % to 28 % buffer B in buffer A, linear for 50 min. In all experiments, the columns were thermostatted at 30 °C.

NMR. NMR spectra were taken on a Bruker AM400 spectrometer operating at 400.13 MHz for protons, interfaced to an Aspect 3000 computer. The samples were dissolved in 90 % H_2O/10 % D_2O (7–15 mg/ml) and the pH adjusted to 3.5 (pH meter reading). All experiments were performed at 25 °C. Spectra were referenced to external 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid (BDH). The solvent resonance was suppressed by presaturation during the relaxation delays. TOCSY spectra were taken using a MLEV-17 mixing sequence with a duration of 60–80 ms. For the NOESY spectra, a mixing time of 400 ms was used. In the NOESY experiments, the solvent resonance was also irradiated during the mixing period.

TOCSY spectra were used for the identification of typical amino acid patterns and NOESY spectra were used for the sequential assignment essentially as described elsewhere (Chan et al., 1989; Slijper et al., 1989).

ESI-MS. Electrospray mass spectra were recorded on a triple quadrupole Perkin-Elmer-Sciex API III mass spectrometer equipped with a nebulizer-assisted electrospray (ion spray) source (Sciex, Thornhill, Toronto, Canada). The mass spectrometer was operated in positive ion mode. Profile spectra were recorded by acquiring data points every 0.3 Da at a scan rate of 6.33 s/scan from *m/z* 400–2200. The mass spectra were an averaged sum of 4–5 scans and contained the mass peaks for the 3–5-fold, in some cases also the 6-fold, protonated molecular ions. The ESI voltage was 4.9 kV; the orifice voltage was +130 V.

The nisin derivatives were dissolved in methanol/1 % formic acid (1:1, by vol.) at a concentration of approximately 100 µg/ml. The solution was introduced into the ESI source at a constant flow rate of 5 µl/min with a syringe infusion pump (Harvard Apparatus, Model 22, South Natick, USA) in combination with a microlitre syringe (100 µl, Hamilton, no. 1710).

Determination of biological activity. The biological activity towards *Micrococcus flavus* DSM1790, *Clostridium tyrobutyricum* B577 and *Streptococcus thermophilus* R_S was determined by an agar-diffusion assay at pH 7.0 (Tramer and Fowler, 1964). Dilution series (five concentrations, dilution factor of 1.8) of the nisin species in 0.05 M sodium phosphate, pH 7.0, were prepared. In each plate, a nisin A standard was used as reference.

Minimal inhibitory concentration (MIC) values were determined by extrapolation of the halo diameter versus log(concentration) plots to a halo of size zero. The ratio of the MIC value of the modified nisin samples relative to nisin A was determined. This parameter showed a better reproducibility than the absolute MIC values.

In the case of [2-hydroxy-Ala5]nisin-(1–32)-peptide amide, the biological activity was also determined by incubating a *Lactococcus lactis* MG1614 culture for 0.5 h at 30 °C in 0.05 M sodium acetate, 0.6 % NaCl, pH 4.0, with different concentrations of either native nisin or the modified nisin. Subsequently, the cultures were diluted and the density of the surviving cell suspensions was determined.

RESULTS

RP-HPLC. Analysis of a commercial nisin preparation showed that it contained several minor components. Fig. 3 shows the ultraviolet trace of an analytical RP-HPLC separation of the nisin sample used for the isolation of the chemically modified nisin components. Six fractions were isolated by preparative RP-HPLC and their chemical structure was determined by two-dimensional NMR spectroscopy and electrospray mass spectrometry as described below. The different components were identified as [2-hydroxy-Ala5]nisin (fraction 1), [Ile4-amide,pyruvyl-Leu6]des-Dha5-nisin (fraction 2), [Met(O)21]nisin (fraction 3), and [Ser33]nisin (fraction 4). Fraction 5 was native nisin A and fraction 6 appeared to be nisin-(1–32)-peptide amide, which has already been described by Chan et al. (1989). An additional component, obtained by freeze drying a solution of nisin A from 0.1 M HCl, was identified as [2-hydroxy-Ala5]nisin-(1–32)-peptide amide.

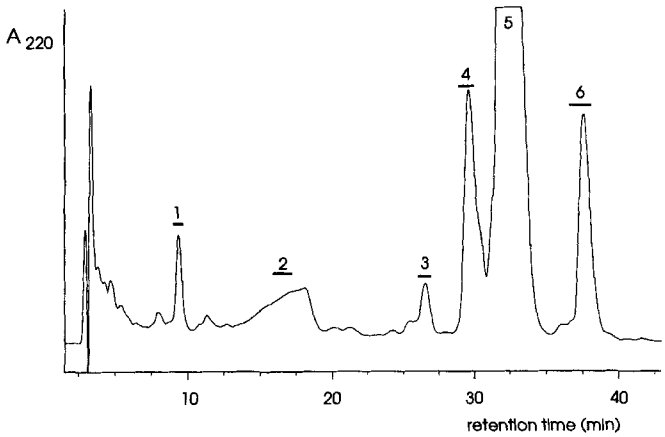

Fig. 3. Analytical RP-HPLC pattern of a commercial nisin preparation used for the isolation of the chemically modified nisin species. Bars denote fractions that were isolated by preparative RP-HPLC.

Mass spectrometry. The molecular masses of the modified nisins were determined by ESI mass spectrometry (Table 1). In several cases, the presence of mass peaks of low intensity with masses 16 Da and 32 Da higher than that of the main component indicated partial oxidation of one or both methionine residues. The calculated isotopic distribution of the molecular ions of nisin showed that the most abundant ion of the cluster has a mass which is approximately 2 Da higher than the monoisotopic mass. This situation is reflected in the ESI mass spectra. The relative molecular masses of the different modified nisins determined experimentally by ESI-MS are about 2 Da higher than the monoisotopic masses (Table 1). Note that three of the modified nisins, [2-hydroxy-Ala5]nisin, [Ile4-amide,pyruvyl-Leu6]des-Dha5-nisin and [Ser33]nisin, have identical molecular masses. ESI mass spectrometry showed that the isolated fractions were sufficiently pure for NMR investigations and biological testing.

NMR. Fig. 4 shows the ^{1}H-NMR spectra of the different nisin species. The vinyl proton resonances originating from the unsat-

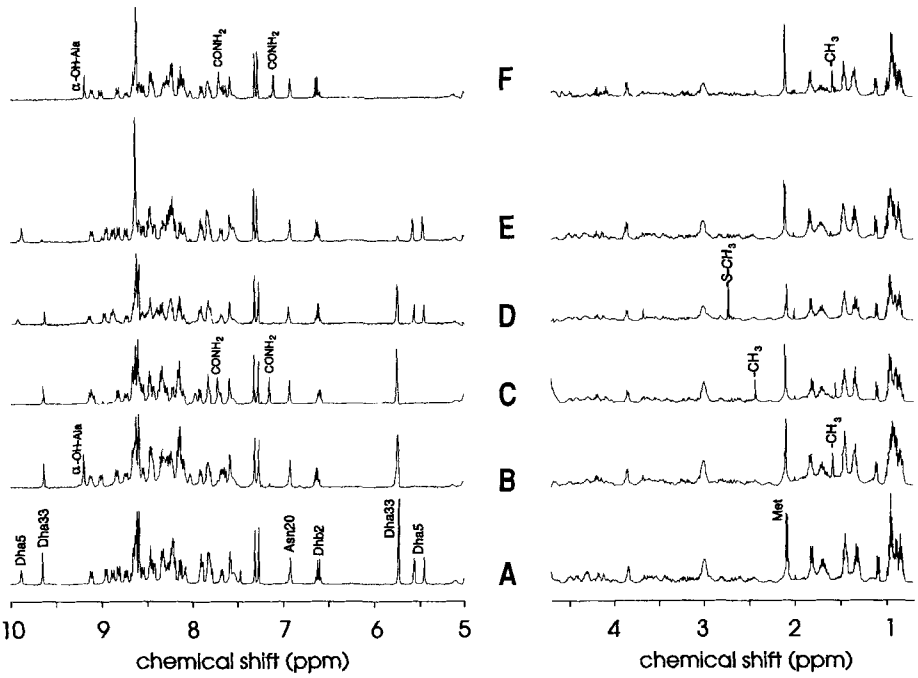

Fig. 4. ^{1}H-NMR spectra of native nisin A (A), [2-hydroxy-Ala5]nisin (B), [Ile4-amide,pyruvyl-Leu6]des-Dha5-nisin (C), [Met(O)21]nisin (D), [Ser33]nisin (E), and [2-hydroxy-Ala5]nisin-(1–32)-peptide amide (F). The assignment of several resonances is given in the figure. Experimental conditions: 10 % D_2O/90 % H_2O, pH 3.5, 25 °C.

Table 1. Characterization of chemically modified nisins by electrospray mass spectrometry (the calculated values constitute the monoisotopic masses).

Compound	Molecular mass	
	calculated	experimental
	Da	
Nisin	3351.6	3353.3 ± 0.5
[2-hydroxy-Ala5]Nisin	3369.6	3371.5 ± 0.4
[Ile4-amide,pyruvyl-Leu6]Des-Dha5-nisin	3369.6	3371.0 ± 0.6
[Ser33]Nisin	3369.6	3371.9 ± 0.3
[Met(O)21]Nisin	3367.5	3369.6 ± 0.1
Nisin-(1−32)-peptide amide	3153.5	3155.3 ± 0.6
[2-hydroxy-Ala5]Nisin-(1−32)-peptide amide	3171.5	3173.2 ± 0.7

urated amino acids are observed between 5 ppm and 6.8 ppm. Their presence or absence provides the first indications on the chemical structure of the derivatives. The ^{1}H-NMR spectra of the modified nisins were completely assigned by analysis of their TOCSY and NOESY spectra (Tables 2 and 3).

Characterization. The chemically modified nisin components were characterized as follows.

[2-hydroxy-Ala5]nisin. In the NMR spectrum of this compound, the vinyl resonances of Dha5 at 5.46 ppm and 5.57 ppm are absent and two singlets at 9.19 ppm and 1.60 ppm are observed (Fig. 4B). In the TOCSY and NOESY spectra, patterns of all residues except Dha5 could be recognized. The singlet at 9.19 ppm was assigned to residue 5. In the NOESY spectrum, a cross-peak is observed between the resonance at 9.19 ppm and the one at 1.60 ppm, which indicates that both resonances belong to the same residue. The fact that the amide resonance of residue 5 is a singlet implies that this residue has no CαH proton. By ESI-MS, a mass 18.2 Da higher than that of native nisin was determined (Table 1). These observations indicate that residue 5 is a 2-hydroxyalanine formed by the addition of a water molecule to Dha5. The missing CαH resonance can easily be explained by the 2-hydroxy group which is not observed by NMR in aqueous solution. The conversion of Dha5 to 2-hydroxyalanine leads to changes in chemical shifts of residues 3−11, which suggests conformational changes in the first two rings (Table 3).

This derivative is an intermediate: it spontaneously converts to [Ile4-amide,pyruvyl-Leu6]des-Dha5-nisin. At pH 3.5 (the pH of the solutions used for the NMR experiments), however, the 2-hydroxyalanine derivative was sufficiently stable to allow for the recording of one two-dimensional NMR spectrum (in approximately 16 h). After each two-dimensional NMR experiment, the sample was repurified by RP-HPLC.

[Ile4-amide,pyruvyl-Leu6]des-Dha5-nisin. In the NMR spectra of this compound, the patterns of Dha5 are missing (Fig. 4C). Additional amide signals at 7.16 ppm and 7.73 ppm, and a singlet at 2.45 ppm, are observed. The resonances at 7.16 ppm and 7.73 ppm were assigned to the -$CONH_2$ group of residue 4 and the singlet at 2.45 ppm to the pyruvyl CH_3 group of residue 6. A mass that was, within experimental error, equal to that of [2-hydroxy-Ala5]nisin was found by ESI-MS. The cleavage at the 2-hydroxyalanine residue into the corresponding peptide amide and pyruvyl peptide, which are still linked by the lanthionine, does not lead to a change in molecular mass.

In NMR analysis, all residues in the (3−11) segment show changes in chemical shift upon the opening of the first ring (Table 3).

Table 2. Assignment of the ^{1}H-NMR spectrum of nisin A. Experimental conditions: 10% D_2O/90% H_2O, pH 3.5, 25°C. Amino acid nomenclature according to IUPAC-IUB recommendations. Ala* and Abu* represent the alanyl- and β-methyl alanyl moieties of lanthionine, respectively. Dha and Dhb represent dehydroalanine and dehydrobutyrine, respectively. In the case of degeneracy of methylene or methyl protons, only one chemical shift value is given.

Residue	Chemical shift			
	NαH	CαH	CβH	other
	ppm			
Ile1	–	4.13	2.12	CγH$_2$ 1.33, 1.57 Cβ′H$_3$ 1.12 CδH$_3$ 1.00
Dhb2	–		6.63	CγH$_3$ 1.85
Ala*3	8.25	4.64	3.15, 3.30	
Ile4	7.85	4.36	2.11	CγH$_2$ 1.19, 1.39 Cβ′H$_3$ 0.97 CδH$_3$ 0.88
Dha5	9.90		5.46, 5.57	
Leu6	8.97	4.44	1.75	CγH 1.71 CδH$_3$, Cδ′H$_3$ 0.92, 0.97
Ala*7	8.25	4.55	2.96, 3.08	
Abu*8	8.89	5.14	3.62	CγH$_3$ 1.34
Pro9		4.45	1.84, 2.47	CγH$_2$ 1.96, 2.20 CδH$_2$ 3.47
Gly10	8.66	3.60, 4.44		
Ala*11	7.93	4.03	3.07, 3.67	
Lys12	8.64	4.35	1.83	CγH$_2$ 1.41, 1.53 CδH$_2$ 1.72 CεH$_2$ 3.02 NεH$_3^+$ 7.57
Abu*13	8.35	4.66	3.63	CγH$_3$ 1.34
Gly14	8.33	4.08, 4.16		
Ala15	8.60	4.25	1.49	
Leu16	8.49	4.32	1.77	CγH 1.68 CδH$_3$, Cδ′H$_3$ 0.92
Met17	7.82	4.67	2.12, 2.30	CγH$_2$ 2.47, 2.65 CεH$_3$ 2.11/ 2.10[a]
Gly18	8.11	3.86, 4.13		
Ala*19	7.70	4.52	3.00	
Asn20	8.56	4.70	2.84	NγH$_2$ 6.95, 7.62
Met21	8.28	4.54	2.02, 2.14	CγH$_2$ 2.54, 2.62 CεH$_3$ 2.11/ 2.10[a]
Lys22	8.44	4.32	1.86	CγH$_2$ 1.45, 1.51 CδH$_2$ 1.73 CεH$_2$ 3.02 NεH$_3^+$ 7.57
Abu*23	8.83	5.01	3.58	CγH$_3$ 1.36
Ala24	8.23	4.67	1.46	
Abu*25	9.13	4.77	3.55	CγH$_3$ 1.47
Ala*26	7.85	3.88	2.71, 3.45	
His27	8.75	4.95	3.10, 3.39	C2H 8.66 C5H 7.34
Ala*28	7.92	4.44	2.73, 3.70	
Ser29	8.47	4.51	3.88	
Ile30	8.15	4.21	1.85	CγH$_2$ 1.17, 1.37 Cβ′H$_3$ 0.88 CδH$_3$ 0.88
His31	8.66	4.78	3.20, 3.24	C2H 8.63 C5H 7.29
Val32	8.35	4.20	2.11	CγH$_3$, Cγ′H$_3$ 0.97
Dha33	9.66		5.76	
Lys34	8.22	4.33	1.82, 1.92	CγH$_2$ 1.47 CδH$_2$ 1.72 CεH$_2$ 3.02 NεH$_3^+$ 7.57

[a] Sequential assignment of CεH$_3$ was not possible.

[Met(O)21]nisin. The NMR spectrum of this component lacks the methionine CεH$_3$ resonance at 2.11 ppm and shows an additional singlet at 2.74 ppm (Fig. 4D). In the TOCSY spectrum, the pattern of Met21 is changed as compared to native nisin. By mass spectrometry, a mass 16.3 Da higher than that of native nisin is found. From this and the chemical shift of the CεH$_3$ resonance at 2.74 ppm, which is close to that of dimethylsulfoxide, it is concluded that residue 21 is a methionine sulfoxide.

[Ser33]nisin. Fig. 4E shows that the resonances of Dha33 are missing. In the TOCSY spectrum, one additional pattern

Table 3. Assignment of the ^{1}H-NMR spectra of the chemically modified nisin species. Only those residues that exhibit chemical shift difference(s) of more than 0.05 ppm as compared to native nisin A are shown. Conditions and nomenclature as in Table 2.

Protein		Chemical shift			
	Residue	NαH	CαH	CβH	other
		ppm			
[2-hydroxy-Ala5]Nisin	Ala*3	8.14	4.58	3.06, 3.26	
	Ile4	7.66	4.22	1.94	CγH$_2$ 1.14, 1.39 Cβ'H$_3$ 0.91 CδH$_3$ 0.83
	α-OH-Ala5	9.19		1.60	
	Leu6	8.12	4.41	1.78	CγH 1.65 CδH$_3$, Cδ'H$_3$ 0.92
	Ala*7	8.28	4.54	2.78, 2.91	
	Abu*8	9.01	5.14	3.61	CγH$_3$ 1.37
	Pro9		4.46	1.88, 2.42	CγH$_2$ 1.94, 2.08 CδH$_2$ 3.48
	Gly10	8.66	3.71, 4.36		
	Ala*11	8.04	4.03	3.04, 3.67	
[Ile4-amide,pyruvyl-Leu6]Des-Dha5-nisin	Ala*3	8.32	4.70	3.00, 3,15	
	Ile4	8.13	4.20	1.94	CγH$_2$ 1.21 Cβ'H$_3$ 0.96 CδH$_3$ 0.96 CONH$_2$ 7.16, 7.73
	Leu6	8.65	4.43	1.74	CγH 1.64 CδH$_3$, Cδ'H$_3$ 0.93 COCOCH$_3$ 2.45
	Ala*7	8.66	4.75	2.99	
	Abu*8	9.07	5.09	3.63	CγH$_3$ 1.35
	Pro9		4.43	1.94, 2.44	CγH$_2$ 2.16 CδH$_2$ 3.43, 3.54
	Gly10	8.60	3.66, 4.40		
[Met(O)21]Nisin	Met(O)21	8.41	4.52	2.17, 2.29	CγH$_2$ 2.95 CεH$_3$ 2.74
	Lys22	8.56	4.32	1.84	CγH$_2$ 1.44, 1.52 CδH$_2$ 1.73 CεH$_2$ 3.03 NεH$_3^+$ + 7.56
	Abu*23	8.89	5.01	3.57	CγH$_3$ 1.35
[Ser33]Nisin	Val32	8.28	4.19	2.09	CγH$_3$, Cγ'H$_3$ 0.94
	Ser33	8.49	4.51	3.88	
	Lys34	8.18	4.27	1.77, 1.86	CγH$_2$ 1.43 CδH$_2$ 1.71 CεH$_2$ 3.02 NεH$_3^+$ 7.57
[2-hydroxy-Ala5]Nisin-(1−32)-peptide amide	Ala*3	8.15	4.57	3.07, 3.27	
	Ile4	7.66	4.22	1.94	CγH$_2$ 1.14, 1.38 Cβ'H$_3$ 0.94 CδH$_3$ 0.84
	α-OH-Ala5	9.20		1.60	
	Leu6	8.12	4.40	1.77	CγH 1.64 CδH$_3$, Cδ'H$_3$ 0.92
	Ala*7	8.28	4.53	2.78, 2.91	
	Abu*8	9.02	5.14	3.61	CγH$_3$ 1.37
	Pro9		4.45	1.89, 2.43	CγH$_2$ 1.95, 2.08 CδH$_2$ 3.49
	Gly10	8.66	3.71, 4.40		
	Ala*11	8.04	4.04	3.04, 3.67	
	Val32	8.25	4.10	2.04	CγH$_3$, Cγ'H$_3$ 0.95 CONH$_2$ 7.12, 7.72

characteristic of serine is observed which could be assigned to residue 33. The molecular mass of this compound is 18.6 Da higher than that of native nisin, which confirms the characterization of this compound as [Ser33]nisin.

[2-hydroxy-Ala5]nisin-(1−32)-peptide amide. This degradation product was formed upon freeze drying of nisin A from 0.1 M HCl. The NMR spectrum does not show the Dha5 and Dha33 resonances (Fig. 4F). Instead, singlets at 9.20 ppm and 1.60 ppm and amide resonances at 7.12 ppm and 7.72 ppm are observed. The singlets at 9.20 ppm and 1.60 ppm were assigned to residue 5 in a way similar to [2-hydroxy-Ala5]nisin. The additional amide resonances were assigned to Val32. In the TOCSY spectrum, the pattern of Lys34 is missing and no additional spin systems are observed. The molecular mass is 17.9 units higher than that of nisin-(1−32)-peptide amide (Table 1). By analogy to [2-hydroxy-Ala5]nisin, this compound is converted into [Ile4-amide,pyruvyl-Leu6]des-Dha5-nisin-(1−32)-peptide amide, and also shows changes in chemical shifts of the residues in the region 3−11 similar to those observed for [2-hydroxy-Ala5]nisin (Table 3).

Stability of 2-hydroxyalanine-containing compounds. The stability of derivatives containing a 2-hydroxyalanine residue is strongly pH dependent (Fig. 5). In the pH range 2−4, these intermediates are relatively stable. Above pH 4, their stability decreases substantially with increasing pH as they are converted to the corresponding des-dehydroalanine derivatives. At pH 7, half-lives in the order of hours or less are observed.

Biological activity. The biological activities of the different nisin species were determined using an agar diffusion assay. The ratios of the MIC values of the different species to that of native nisin are presented in Table 4. Modifications in the C-terminal part of the molecule appear to have little or no influence on the biological activity of nisin. However, modifications in the first ring have a strong effect. The formation of 2-hydroxyalanine at position 5 decreases the biological activity considerably. Since the agar diffusion assay is performed at pH 7.0, the experimentally determined MIC values for the 2-hydroxyalanine-containing species could represent those of the corresponding des-dehydroalanine compounds, because of the instability of 2-hydroxyalanine at about pH 7 (Fig. 5). For this reason, the biological activity of [2-hydroxy-Ala5]nisin-(1−32)-peptide amide towards *Lactococcus lactis* MG1614 was determined by incubating the cells with this component at pH 4.0 for 0.5 h followed by a determination of the viable cell density. The concentration

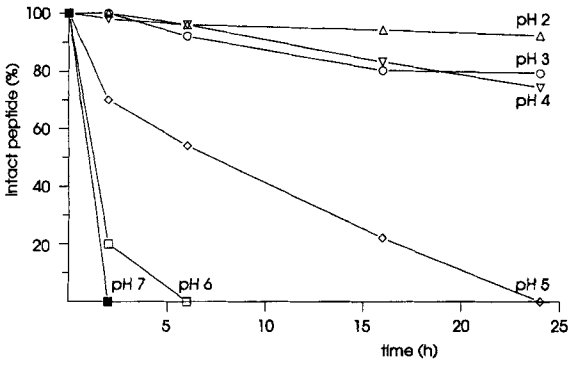

Fig. 5. Stability of [2-hydroxy-Ala5]nisin-(1–32)-peptide amide as a function of pH at 30 °C. The percentage of the intact compound, as judged by the peak area in RP-HPLC, is plotted as a function of incubation time. Protein concentration: 1 mg/ml.

Table 4. The ratio of the MIC values of the modified nisins and the MIC value of native nisin A. The data were obtained from an agar-diffusion assay.

Modified nisin	$MIC_{modified\ nisin}/MIC_{nisin\ A}$		
	M. flavus	*S. thermophilus*	*C. tyrobutyricum*
[Ser33]Nisin	1	1	1
[Met(O)21]Nisin	2	2	3
Nisin-(1−32)-peptide amide	1	0.5	2
[2-hydroxy-Ala5]Nisin	150	40	10
[Ile4-amide,pyruvyl-Leu6]Des-Dha5-nisin	60	30	10
[2-hydroxy-Ala5]Nisin-(1−32)-peptide amide	80	30	10
[Ile4-amide,pyruvyl-Leu6]Des-Dha5-nisin-(1−32)-peptide amide	160	30	10

required to kill 90 % of the *Lactococcus lactis* MG1614 cells was 50 μg/l for native nisin and 20 mg/l for [2-hydroxy-Ala5]nisin-(1−32)-peptide amide, which demonstrates that the 2-hydroxyalanine residue at position 5 leads to a dramatic reduction of the biological activity.

DISCUSSION

The characterization of chemically modified nisin derivatives provides useful information concerning the relation between the chemical structure and biological activity of the bacteriocin. Two nisin degradation products, nisin-(1−32)-peptide amide and [Ile4-amide,pyruvyl-Leu6]des-Dha5-nisin-(1−32)-peptide amide, were described by Chan et al. (1989). In the present paper, we describe [Ile4-amide,pyruvyl-Leu6]des-Dha5-nisin, [2-hydroxy-Ala5]nisin, [2-hydroxy-Ala5]nisin-(1−32)-peptide amide, [Met(O)21]nisin, and [Ser33]nisin.

Nisin appears rather susceptible to chemical modification at its two dehydroalanine residues at positions 5 and 33. In addition, oxidation of methionine residues can occur. The dehydroalanine residues undergo acid-catalyzed addition of a water molecule to the Cα-Cβ double bond, leading to the formation of a metastable 2-hydroxyalanine residue, which ultimately is converted to the corresponding peptide amide and pyruvyl peptide (Gross, 1977). All known chemically modified nisin derivatives contain an intact Dhb2 residue, which indicates that dehydrobutyrine is much more stable than dehydroalanine. It has been demonstrated that [Dhb5]nisin Z is resistant to acid-catalysed addition of a water molecule at position 5 (Rollema et al., 1995). Oxidation of methionine residues is known to occur at low pH (Brot and Weissbach, 1991). Formation of most of the chemically modified nisins could occur at some step in the isolation procedure, where the material is exposed to low pH. [Ser33]-nisin is not formed under these conditions. Its occurrence could be due to incomplete post-translational modification.

Replacement of Dha33 by serine has little or no influence on the biological activity of nisin. This observation confirms the conclusion of Chan et al. (1989) that the removal of the two C-terminal residues has no effect on the biological activity of nisin. Oxidation of Met21 has a relatively small effect on the functional properties of nisin. Dha5 appears to be very important for the biological activity of nisin. Replacement of Dha5 by Dhb reduces the growth-inhibiting effect of nisin by a factor of 2−10 (Kuipers et al., 1992). Formation of a 2-hydroxyalanine residue at position 5 and cleavage of the polypeptide chain at this position reduce the biological activity even more strongly. However, the degree of reduction can vary considerably depending on the target organism. In the case of *M. flavus*, the activity is reduced by a factor of 150 for [2-hydroxy-Ala5]nisin and by a factor of 60 for [Ile4-amide,pyruvyl-Leu6]des-Dha5-nisin. For *C. tyrobutyricum*, both compounds show a reduction in activity by only a factor of 10 (Table 4). The observation that the presence of a 2-hydroxyalanine residue at position 5 reduces the growth-inhibiting effect of nisin seems to be in contrast with the observation of Liu and Hansen (1992) that [Ala5]subtilin, a bacteriocin closely related to nisin, exhibits an unimpaired growth-inhibiting activity but a strongly reduced spore-killing effect. In the case of nisin, [Ala5]nisin and [Ala5, Ala33]nisin were reported to have an unimpaired growth-inhibiting activity (Dodd and Gasson, 1994; Kuipers et al., 1996). The three-dimensional structure of nisin in water and in membrane-mimicking micelles shows a clearly amphipathic character for the N-terminal part of the molecule (van de Ven et al., 1991; van den Hooven et al., 1996a). The hydrophobic residues in this part of the molecule appear to interact with the micelle; they are immersed in the micelle, while the hydrophilic residues are orientated outward (van den Hooven et al., 1996b). In the first ring of nisin, the side chains of Dha5 and Leu6 interact with the micelle. Introduction of the polar 2-hydroxyalanine residue at position 5 could lead to a hampered membrane interaction and consequently to a lowered biological activity. Introduction of the hydrophobic alanine residue at position 5 would not alter the interaction with the membrane and thus would not affect the growth-inhibiting activity. The reduced spore-killing effect of [Ala5]subtilin can be accounted for by assuming that spore-killing and growth-inhibition do not rely on the same mechanism (Liu and Hansen, 1992).

It is striking that the chemically modified nisins that exhibit a reduced biological activity also show changes in chemical shift for residues in a relatively large segment of the molecule (residues 3−11). In case of the des-dehydroalanine derivative, this effect seems trivial: the changes in chemical shift may be due to the opening of ring A. The changes in chemical shift of the 2-hydroxyalanine derivative differ significantly from those observed for the des-dehydroalanine compound. The changes in chemical shift observed for the 2-hydroxyalanine derivative could reflect changes in conformation of rings A and B, which might contribute to a weakened interaction with the bacterial membrane.

In conclusion, the occurrence of chemically modified nisin species demonstrates the limited chemical stability of nisin. The altered biological activity of these compounds illustrates the im-

portance of Dha5 as a hydrophobic moiety in an amphipathic region and the integrity of the first ring in nisin for its functional properties.

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Note added in proof. After the submission of this paper, we learned that a related study had been performed by Cruz et al. [Cruz, L., Garden, R. W., Kaiser, H. J. & Sweedler, J. V. (1996) Studies of the degradation products of nisin, a peptide antibiotic, using capillary electrophoresis with off-line mass spectrometry, *J. Chromatogr. A735*, 375–385].